A COUPLED TRANSCRIPTION–TRANSLATION SYSTEM DERIVED FROM *ESCHERICHIA COLI*: THE USE OF IMMOBILIZED DEOXYRIBONUCLEASE TO ELIMINATE ENDOGENOUS DNA

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1. Introduction

The recent development of cell-free systems capable of translating a variety of messages with high fidelity has permitted the identification of the gene products of many DNAs. We hoped that a similar approach would be applicable to mtDNA of yeast. For this purpose we chose a system derived from *Escherichia coli,* since not only have several successful systems been described in the literature (e.g. [1,2]), but a high fidelity of recognition might be expected on the basis of the many similarities between prokaryotic and mitochondrial genetic systems [3]. In initial experiments, however, we discovered that *E. coli* extracts prepared according to published procedures [4] still contained appreciable amounts of endogenous DNA, resulting in poor response to added template. We overcame this problem by the development of a rapid and considerably simplified procedure for preparation of *E. coli* cell-free extracts, in which endogenous DNA is removed by incubation of the extracts with pancreatic deoxyribonuclease immobilized on Sepharose. Extracts prepared in this way carry out efficient and accurate transcription and translation of bacteriophage DNA and respond well to mtDNAs from various sources. The system will no doubt be of great use for the in vitro transcription–translation of other DNA templates.

Abbreviations: SDS, sodium dodecylsulphate; TCA, trichloroacetic acid

2. Methods and materials

2.1. *Preparation of S30 extracts of* E. coli

A modified procedure of Nirenberg and Matthaei [5] was employed. *E. coli* (Ka 81) was grown in 1% Bacto-trypton, 1% NaCl, 0.5% yeast extract and 0.1% glucose (pH 7.0) [6] to an $A_{550\,nm}$ of 0.7, cooled rapidly by adding frozen 0.9% NaCl (1 litre ice/2.5 litre medium), was harvested by continuous flow centrifugation at 17 000 × *g* at 4°C (flow rate 200 ml/min) and washed once with 50 mM NH_4Cl, 10 mM Mg-acetate, 10 mM Tris–HCl (pH 7.5). All further steps were performed under ribonuclease-free conditions. Cells were stored at −20°C until use. Frozen cells were allowed to thaw completely at 0°C and ground vigorously for 5 min in a mortar with Alcoa (2.5 g/g cells). The resulting paste was suspended in 10 mM Mg-acetate and 10 mM Tris–HCl (pH 7.5) and centrifuged for 10 min at 15 000 × *g* and at 2°C. After the addition of 2-mercaptoethanol to a final concentration of 6 mM, the supernatant was centrifuged for 30 min at 30 000 × *g*. The concentration of NH_4Cl in the supernatant resulting from this centrifugation was then brought to 50 mM by addition of a 1 M solution and incubation for 40 min at 37°C was carried out with about 0.1 ml packed deoxyribonuclease-Sepharose beads (vide infra) per ml supernatant. The deoxyribonuclease–Sepharose beads were removed by a 3 min centrifugation at 3000 × *g*. Overnight dialysis against two changes of 50 mM NH_4Cl, 10 mM Mg-acetate, 10 mM Tris–HCl (pH 7.5) and 6 mM mercaptoethanol was carried out at 4°C

and small aliquots were stored in liquid nitrogen until needed.

2.2. *Preparation of deoxyribonuclease–Sepharose*

Deoxyribonuclease–Sepharose was prepared essentially according to the procedure described by Cuatrecasas [7]. 10 ml packed Sepharose 4B (Pharmacia) was activated with 500 mg cyanogen bromide at pH 10.0–10.5 and washed with 150 mM NaCl, 50 mM NaP_i (pH 7.4), 5 mg deoxyribonuclease (ribonuclease-free; Worthington) was then added and coupling was carried out overnight at 4°C. The deoxyribonuclease–Sepharose beads were washed with phosphate-buffered saline (90% of the deoxyribonuclease was coupled on $A_{280\ nm}$ basis), remaining active groups were blocked by incubation for 4 h at room temperature with 0.5 M ethanolamine (pH 9.0), washed again with phosphate-buffered saline and stored in a total volume of 25 ml in the presence of 0.02% sodium azide at 4°C. The preparation shows high stability: no loss of activity has been noted even after two years storage at 4°C. Before use, the deoxyribonuclease–Sepharose beads were washed three times with 50 mM NH_4Cl, 10 mM Mg-acetate and 10 mM Tris–HCl (pH 7.5). Activity of the deoxyribonuclease–Sepharose was checked by incubating bacteriophage T_7 DNA (100 μg/ml) with 0.1 ml packed beads per ml for 40 min at 37°C in 5 mM Mg-acetate, 1 mM EDTA and 10 mM Tris–HCl (pH 7.5). Integrity of the DNA was analysed on 0.5% agarose gels containing 0.5 μg ethidium bromide per ml [8]. Bands were visualized by their fluorescence (ethidium–DNA complex) on a short-wavelength ultraviolet lamp. Gels of phage T_7 DNA incubated with deoxyribonuclease–Sepharose showed no fluorescence at all, indicating that all DNA was indeed degraded (up to 3 μg DNA was applied per gel). Control phage T_7 DNA incubated in the presence of Sepharose had the same electrophoretic mobility as untreated DNA (0.2 μg DNA was applied per gel).

2.3. *Protein synthesis in* E. coli *S30 extracts*

Incubations were for 15 min at 36°C in 150 mM NH_4Cl, 10 mM Mg-acetate, 50 mM Tris–HCl (pH 7.8), 1 mM ATP, 0.25 mM each of GTP, UTP and CTP, 5 mM phosphoenolpyruvate, 20 μg/ml pyruvate kinase, 1 mM dithiothreitol, 6 mM 2-mercaptoethanol, 0.2 mM leucovorin (folinic acid), Ca^{2+} salt (Serva, Heidelberg), *E. coli* S30 extract (5 mg protein/ml) and 0.05 mM each of the 20 amino acids (minus methionine). The concentration and specific activity of [^{35}S]methionine and the quantities of exogenous DNA, when added, are indicated in the legends. 10 μl samples were spotted on Whatman 3 MM paper discs, which had been sprayed beforehand with 5% TCA and methionine (1 mg/ml) and dried. The discs were then washed for 15 min successively in: 5% TCA at room temperature, 5% TCA at 95°C, ethanol/ether (1:1) at room temperature, ether at room temperature, dried and scintillation counted. For electrophoretic analysis the reactions were stopped by addition of 1/3 volume 12.5% SDS, 25 mM dithiothreitol, 20% (w/v) glycerol and 50 μg/ml bromophenol blue and boiled for 2 min.

3. Results

Of all procedures so far described for the preparation of a cell-free transcription–translation system from *E. coli* only one, that originally described by Gold and Schweiger [4], makes any attempt to remove endogenous DNA. Crucial steps in their procedure appear to be extensive fragmentation of DNA by breakage of the cells by homogenization with glass beads, so that this is recovered in the S100 supernatant after centrifugation of the ribosomes. Nucleic acids are subsequently removed from the S100 by chromatography on DEAE–cellulose. As Table 1 shows, however, the combination of this S100 with washed ribosomes still shows measurable protein synthetic activity in the absence of added DNA, presumably due to the contamination of the ribosome fraction with DNA fragments. As a consequence, we find that stimulation of [^{35}S]methionine incorporation by bacteriophage T_7 DNA, although comparable with that reported by O'Farrell and Gold [10], is low and response to yeast mtDNA is marginal. This poor response was, further brought out by electrophoretic analysis of the products synthesized (data not shown): no new specifically mitochondrial products could be detected over the high background of endogenously-synthesized material.

Table 2 shows that the endogenous activity observed is indeed due to DNA, since it can be eliminated by preincubation of the extracts with pancreatic deoxy-

Table 1
Amino acid incorporation by the Gold and Schweiger system programmed by phage T_7 DNA and yeast mtDNA

Experiment	Additions	Incorporation ^{14}C or ^{35}S (cpm/10 μl)
1	None	698
	Phage T_7 DNA (12.5 μg/ml)	2880
2	None	15 286
	Yeast mtDNA (35 μg/ml)	20 510

Preparation of ribosomes and supernatant and conditions of protein synthesis were as described by Gold and Schweiger [4]. Incubations were for 20 min at 37°C. In experiment 1, 4 μM [^{14}C]phenylalanine (500 mCi/mmol) was used and in experiment 2, 2 μM [^{35}S]methionine (7 Ci/mmol). Published procedures were used for the preparation of phage T_7 DNA [9] and mtDNA from *Saccharomyces cerevisiae* JS1-3D [8].

ribonuclease. As might be expected, such extracts respond poorly to added DNA templates, due to the continued presence of the enzyme. However, preincubation with deoxyribonuclease covalently coupled to Sepharose permits effective removal of endogenous DNA, while response to added DNA is retained, since the deoxyribonuclease – by virtue of its immobilization on a solid support – can be removed by a simple centrifugation step. For bacteriophage T_7 DNA, total activity found corresponds to about 7 μg newly-synthesized protein per ml incubation mixture, which is equivalent to that reported by O'Farrell and Gold [10], using DEAE-treated S100 plus ribosomes, but endogenous activity is only 1.6% of this. Activity is apparently not limited by the rate of transcription, since addition of extra purified *E. coli* RNA polymerase is without effect.

The effectiveness of removal of endogenous DNA by the preincubation procedure is further illustrated by electrophoretic analysis of the products synthe-

Table 2
Characteristics of protein synthesis by different *E. coli* extracts programmed by phage T_7 DNA

Pretreatment S30	Incorporation (cpm/10 μl)		Stimulation due to viral DNA
	Endogenous control	Phage T_7 DNA (50 μg/ml)	
None	18 477	59 175	2.2
Sepharose	18 079	64 983	2.6
Deoxyribonuclease	534	18 455	33.6
Deoxyribonuclease–Sepharose	754	47 671	63.0

The supernatants of the 30 000 × *g* centrifugation (S30) were incubated for 40 min at 37°C in the presence of Sepharose, deoxyribonuclease (3 μg/g cells) and deoxyribonuclease–Sepharose, respectively, as indicated in the table. Conditions of protein synthesis were as described in Materials and methods. [^{35}S]Methionine (1.4 Ci/mmol) was 20 μM. Hot TCA-insoluble radioactivity was scintillation counted at 50% efficiency. Values given are the means of duplicate determinations and have been corrected for a zero time control of 653 cpm. Stimulation has been calculated as the increase of incorporation due to viral DNA compared with the endogenous control.

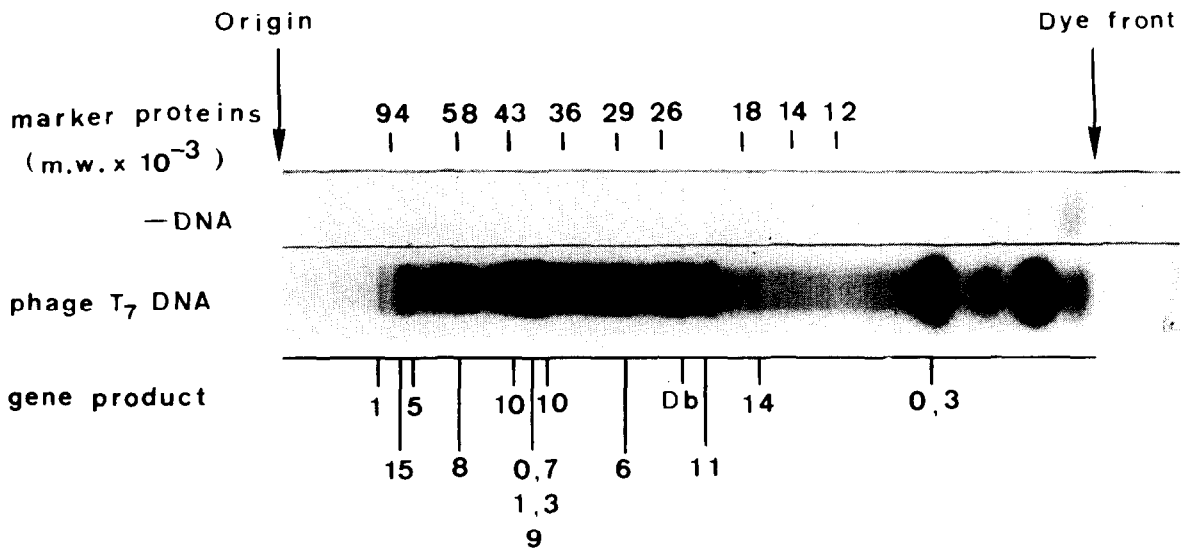

Plate 1. Autoradiogram of [^{35}S]methionine-labelled polypeptides of a phage T_7 DNA programmed protein synthesis, fractionated on a SDS 15% polyacrylamide slab gel [11]. After electrophoresis the gel was sliced length-wise to give two sections of equal thickness. One section was washed overnight in 5% TCA, followed by a 1 h wash in water and dried for 24 h autoradiography (film Structurix D10, Agfa-Gevaert). The other section was stained with Coomassie brilliant blue [12] and destained with 10% methanol, 10% acetic acid to visualize the marker proteins. Standards of known molecular weight included phosphorylase a (94 000), catalase (58 000), ovalbumin (43 000), lactate dehydrogenase (43 000), carbonic anhydrase (29 000), α-chymotrypsinogen (26 000), β-lactoglobulin (18 000), ribonuclease A (14 000) and cytochrome *c* (12 400). Phage T_7 DNA gene products have been indicated on the basis of molecular weights as given by Studier [13]. D.b., DNA binding protein.

sized. Plate 1 shows that with application of equivalent amounts of extract to SDS–acrylamide gels, phage T_7-specific proteins are clearly visible after only 24 h exposure, whereas no product can be detected in the absence of DNA. Only after an exposure of at least 14 days do bands with M_r 68 000 and 26 000 become visible, together with some heterogeneous material near the dye front.

Table 3 shows that extracts prepared according to this procedure are highly active in the coupled transcription–translation of mtDNAs from various sources, with stimulation factors ranging from 13-fold for yeast mtDNA to 28-fold for rat- and calf-liver mtDNAs. Chick-liver mtDNA was also found to be

Table 3
[^{35}S]Methionine incorporation by the *E. coli* extract programmed by different mtDNAs

Additions	Incorporation (cpm/10 μl)	Stimulation due to mtDNA
None	255	–
Yeast mtDNA (50 μg/ml)	3555	13
Rat-liver mtDNA (30 μg/ml)	7454	28
Calf-liver mtDNA (30 μg/ml)	7272	28

Conditions of protein synthesis were as described in Methods. [^{35}S]Methionine (9 Ci/mmol) was 4 μM. Values have been corrected for a zero time control of 173 cpm. Published procedures were used for the preparation of yeast mtDNA [8] and rat and calf-liver mtDNA [14].

active (data not shown). Stimulation is to some extent dependent on the integrity of the template, since rat-liver mtDNA preparations consisting largely of components II and III stimulated activity to a lesser extent. In all cases, however, the response to mtDNA was superior to results reported previously [15,16]. All mtDNAs direct the synthesis of material banding in SDS–acrylamide gels in the range M_r 5000–100 000. At present, however, analysis of the relationship of this material to known products of mitochondrial protein synthesis is complicated by a tendency of the in vitro synthesized polypeptides to aggregate specifically with *E. coli* proteins and it is not yet clear whether correct and complete synthesis of mitochondrial proteins is occurring. In the case of yeast mtDNA, use of an indirect immunoprecipitation technique has shown that antigenic determinants of cytochrome *c* oxidase and of the cytochrome bc_1 complex are among the products of in vitro synthesis [17]; further characterization of these products is in progress.

4. Discussion

We have found that *E. coli* extracts can be depleted of endogenous DNA by preincubation with pancreatic deoxyribonuclease immobilized on Sepharose. Such extracts respond better to added DNA templates than do extracts prepared according to previously published procedures [1,10]. They can, therefore, be used with advantage for the in vitro transcription–translation of heterologous DNAs. The preincubation procedure has the additional advantages that it is simple, rapid and makes steps involving elimination of endogenous DNA by chromatographic procedures (cf. [1]) in which variable losses of activity can occur [10] unnecessary. Furthermore, there is no need for the addition of extra purified RNA polymerase, sometimes necessary in the Gold and Schweiger system [4].

The cell-free system we have developed functions with high fidelity as far as can be judged from the examination of the products synthesized under the direction of phage T_7 DNA: not only can many early gene products such as RNA polymerase (gene 1; M_r 100 000), a DNA ligase (gene 1.3; M_r 40 000) and the gene 0.3 product (M_r 8700) be identified on the basis of their mobility in SDS–acrylamide gels (Plate 1; [13], but bands corresponding to many late products are also present. In this respect, the system compares well with the in vivo situation and with in vitro systems as described previously by Hopper et al. [18]. It is interesting to note, however, that whereas other authors [19,20] have reported significant synthesis in vitro of the late gene product, lysozyme, the present system like that of O'Farrell and Gold [10], gives neither material banding in SDS–acrylamide gels at a position expected for lysozyme, nor a significant amount of enzymically-active protein (data not shown).

So far we have found that mtDNAs from a variety of sources are active in directing transcription–translation in this system and that yeast mtDNA gives rise to the synthesis of antigenic determinants of known products of mitochondrial protein synthesis, thus establishing that the genes for these determinants are indeed located on mtDNA [17]. Further characterization of the products synthesized and mapping of the genes responsible for activity is now in progress and the results of this will be reported in detail elsewhere.

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